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Review

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Biological applications of the electrochemical sensing of nitric oxide: fundamentals and recent developments

Abstract: Nitric oxide (NO) is a unique cellular messenger linked to a number of important biological processes. Its free radical nature, small size and fast diffusivity make it highly reactive and membrane permeable. Unfortunately, its reactivity, coupled with the inherent complexity of *in situ* biological measurements, makes it a challenge to detect. For the past 20 years, electrochemical methods have been used to investigate the role of NO in a number of biological processes, including vascular physiology, immune response, neuronal mediation, tissue growth and oxidative stress. This review examines the biological applications of electrochemical NO sensors and the technologies used to elucidate different physiological phenomena associated with this unique biomolecule with a specific focus on the developments and innovations reported in the last 3 years.

Keywords: angiogenesis; bio-electrochemical methods; *in situ* assays; immune response; neuronal communication; nitric oxide.

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Introduction: biological role of nitric oxide

Nitric oxide (NO) is a ubiquitous biological mediator that is involved in a wide range of physiological functions (Moncada et al., 1991). Historically, this compound was initially identified as the endothelial-derived relaxing factor by Furchgott (Khan and Furchgott, 1987), Moncada (Palmer et al., 1987) and Ignarro (Ignarro et al., 1987). NO is a product of an enzymatic-catalyzed reaction between the substrate, L-arginine, and oxygen resulting in the formation of a molecule of NO and a molecule of L-citrulline (Palmer et al., 1988; Moncada and Higgs, 1993). This reaction is performed by a specific family of enzymes known as NO synthases (NOSs) (Moncada et al., 1991). There are three isoforms of NOS: endothelial, neuronal (nNOS) and inducible (Stuehr, 1999). All of these enzymes are inhibited by N(G)-nitro-L-arginine methyl ester (L-NAME), an analogue of L-arginine (Rees et al., 1990). Specific inhibitors of each isoform are commercially available and are routinely used to highlight the role of a specific NOS isoform.

This review focuses on the electrochemical detection of NO in biological samples. A variety of reviews have already been carried out in this field (Bedioui et al., 1996; Malinski et al., 1996; Bedioui and Villeneuve, 2003; Bedioui et al., 2010; Privett et al., 2010; Patel, 2011). In this review, the fundamental principles and challenges associated with bio-electrochemical sensing of NO are described. Novel techniques and designs manufactured to mitigate these challenges are also reviewed. Due to the sheer volume of published material on this specific topic, an exhaustive review encompassing the whole field would be excessive. Instead, the aim of this work is to provide a broad overview of the possibilities offered by electrochemical detection applied to biological studies. A specific focus is given to studies providing significant and novel understanding of NO-related biochemistry. Therefore, reports focusing solely on the electrochemistry of NO, without addressing its biological relevance, are not specifically considered. Overall, the potential uses of NO sensors in vascular, neuronal and immune physiologies are reviewed, with a specific highlight on recent and innovative developments of the technology published over the past 3 years.

Electrochemical detection of nitric oxide

In situ detection of NO is often challenging because of the low levels and short lifetime of this radical in the biological environment. The use of the NOS inhibitor, L-NAME, is one of the simplest ways to show the action of NO in a given biological system (or pathway), however this method is often difficult to quantify. The high biological significance of NO has motivated the development of several methods of quantitative analytical detection. A large number of these methods of detection involve fluorescent-based biomarkers paired with highly sensitive spectroscopic detection schemes (Archer, 1993; Ye et al., 2008; Hetrick and Schoenfisch, 2009). For example, diaminofluoresceins (DAF) have been proposed as selective and quantitative fluorescent-based NO biomarkers (Kojima et al., 1998; Ye et al., 2009). Another popular method, the Griess test, monitors the levels of nitrite, the by-product of NO oxidation (Ignarro et al., 1993; Nims et al., 1996). The oxidation of the colorless 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to the green $ABTS^{+}$ by NO can be detected spectrophotometrically (Nims et al., 1996). Unfortunately, most of these methods are expensive, time consuming and label-based. Electron paramagnetic resonance spectrometry has also been used to selectively detect and quantify NO *in vivo* after adduction by an iron-containing complex. This method allows for the *in situ* detection of NO in different organs within the same organism (Yoshimura et al., 1996).

Electrochemical methods are routinely used for bioanalytical studies. Electrochemical sensors are cheap, easy to manufacture with bench-top methods, amenable to miniaturization and can be chemically modified to address a wide range of biochemical questions. These sensors initiate an electrochemical reaction at the surface of an electrode. The electrode is held at a potential compatible with this reaction and the current produced is measured. Once processed, this current can be related to the absolute level of the analyte, or to the variations in its concentration (Bard and Faulkner, 1980). The reaction can be controlled by modulating the electrode potential. The standard experimental procedure, amperometry, uses a fixed potential. Potentiodynamic methods, where the input potential is varied, are also applicable to sensing. The potential waveform can be tuned to improve the sensors selectivity and stability (Bedioui and Trévin, 1998). Another advantage of electrochemical detection is its fast acquisition speed, with measurement resolutions down to a fraction of a millisecond. Most of the electrochemical probes are in the range of a few micrometers to tens of micrometers in size, which is ideally suited for biochemical studies.

Initially, *in situ* detection of NO via electrochemical detection was performed using a nickel porphyrin/nafion-modified carbon fiber microelectrode (Malinski and Taha, 1992). The ‘electrocatalytic’ mechanism of this coating is still unclear (Ciszewski and Milczarek, 2003). It has been proposed that this polymer modifies the electrode surface without interacting with the reaction or improves the selectivity by changing the redox potential. Furthermore, the activity of the sensor is unaltered by the absence of nickel or porphyrin. NO sensors typically monitor NO concentration via its one electron oxidation. The reduction of NO has also been investigated; however this method is especially challenging due to direct interferences with the reduction of oxygen.

The ability to selectively detect NO with a high specificity must be considered when developing and characterizing a sensor. The high chemical complexity of the biological background raises another challenge to *in situ* electrochemical sensing. The analyte of interest, here NO, is diluted in a biological matrix containing cells, macromolecules, salts, dissolved gases and other mediating molecules. As several of these compounds are electroactive, they can interfere with NO detection. Endogenous nitrite poses a particular problem to NO detection since it oxidizes within the same potential range as NO. Selectivity is often achieved by adjusting the potential waveform, or more often by coating the sensor with selective membranes, in particular the polymer nafion (Brown et al., 2009). The components of the biological matrix pose another threat to *in situ* electrochemical detection; these molecules can potentially inactivate the sensor by adhering to its surface, thus decreasing its efficacy and stability. This phenomenon is known as biofouling (Wisniewski and Reichert, 2000; Wisniewski et al., 2000). In the specific case of *in vivo* sensing, false measurements or failure of the sensor can be caused by coagulation, immune response and device encapsulation due to a foreign body reaction. In these cases, membrane coatings can mitigate biofouling by preventing the fouling molecules from interacting with the surface of the sensor or by improving bio-integration (Mowery et al., 2000; Frost et al., 2005; Trouillon et al., 2009a,b).

Commercial Clark-type electrodes, typically modified with a poly(tetrafluoroethylene) (PTFE) membrane, are available in various sizes, thus allowing for the study of a wide range of samples, and have been routinely used for biological studies (Sud et al., 2008; Teng et al., 2008; Yang et al., 2008). Despite the usefulness and convenience of NO commercial sensors, this review focuses on the development and design of custom NO sensors. Most of the NO sensors reported are based on modified carbon fiber microelectrodes (Pontié et al., 2000; Brunet et al., 2003). These electrodes consist of a section of carbon fiber insulated in a pulled glass capillary and sealed with epoxy. To improve their selectivity towards NO, an active layer (such as platinum black, poly-eugenol, porphyrinic polymers, or nafion) is deposited on the carbon electrode surface. Similarly, modified platinum microelectrodes can also be used (Shin et al., 2008; Park et al., 2009). Other forms of carbon materials have been investigated, including nanotubes and nanoparticles (Zheng et al., 2008; Porras Gutierrez et al., 2009; Deng et al., 2010; Xu et al., 2011; Chng and Pumera, 2012), graphene and nanosheets of graphite (Wu et al., 2010; Ng et al., 2011; Chng and Pumera, 2012), and boron-doped diamond (BDD) electrodes (Patel et al., 2008b, 2010b; MacEachern et al., 2011). Microfabrication has also been extensively used to produce NO sensors in the form of microelectrode arrays (Oni et al., 2004; Chang et al., 2005; Patel et al., 2008a; Trouillon et al., 2010a, 2012; Quinton et al., 2011) or microfluidic systems with integrated electrochemical detection (Amatore et al., 2007; Cha et al., 2010; Gunasekara et al., 2012). The format of the electrochemical sensor has to be adapted for the specificities of the biological sample (cell, tissue, living animal, blood, etc.). Furthermore, the electrochemical detection of NO by-products, such as nitrite, peroxynitrite (Quinton et al., 2011) or S-nitrosothiols (RSNO; Cha et al., 2005, 2009; Cha and Meyerhoff, 2006; Hwang et al., 2008), have also been reported.

The remaining sections of this review focus on the application of bio-electrochemical sensing of NO in the cases of:

- vascular physiology, tissue growth and angiogenesis;
- neuronal communication; and
- immune response and oxidative stress.

The different methods reviewed in this article to elucidate NO physiology in specific samples (cells, excised tissue samples, *in vivo*, etc.) are summarized in Table 1.

Vascular physiology, tissue growth and angiogenesis

One of the main roles of NO *in vivo* is regulating arterial tone and controlling blood pressure. In this case, the release of NO is mechanically triggered by shear-stress at the surface of endothelial cells. The NO released induces the dilation of the smooth muscles lining the artery wall *via* soluble guanylyl cyclase activation (Moncada et al., 1988). The synthesis of NO can also be induced by angiogenic factors and is then involved in angiogenesis, tissue growth and repair (Murohara et al., 1998). NO was reported to inhibit apoptosis and to increase the permeability of the artery wall, thus enabling vascular sprouting and development. Its role in proliferation and angiogenesis means that NO has been identified as a central component of carcinogenesis and tumor malignancy (Ohshima and Bartsch, 1994).

In vitro cell measurements

In 1997, Bedioui and coworkers reported the electrochemical detection of NO in cultured human umbilical vein endothelial cells (HUVEC; Bedioui et al., 1997). In this work, a carbon fiber microelectrode, modified with electropolymerized nickel tetrasulfonated phthalocyanine (NiTSPc) and nafion, was positioned above a layer of HUVECs. Histamine was used to stimulate the release of NO from these cells. Cells pre-incubated with a NOS inhibitor were not effectively stimulated by histamine. Overall this experiment demonstrates that electrochemical detection can successfully detect histamine-induced NO release in HUVECs.

This strategy of positioning a sensor above a cell monolayer led to the application of scanning electrochemical microscopy (SECM) in NO detection *in vitro*. As shown in Figure 1A, this technique involves scanning an electrode over the sample. The spatial distribution of NO release or consumption can therefore be determined. The scanning nature of this technology allows for the precise positioning of the electrode in the three dimensions in space and enables the precise resolution of the sample's geometry. In addition, this technical set-up allows for *in situ* sensor preparation, and more specifically as the stage is displaced it allows the electrode to be immersed in different successive solutions (Pailleret et al., 2003). The electrode can therefore be reproducibly placed in the vicinity of the cell, and NO release triggered by bradykinin can be detected, as shown in Figure 1A.

This technique was later improved by adding a second electrode to the sensor (Isik et al., 2004). This dual electrode sensor had one large electrode (with a diameter of 50 μm) modified with Ni(4-N-tetramethyl) pyridyl porphyrin (NiTmPyP) for NO detection, and one small unmodified distance electrode (with a diameter of 10 μm) used to measure the reduction of molecular oxygen. In this experiment, the reduction of dissolved oxygen was used as a standard when measuring the position of the electrode. This feedback signal was used to control the distance between the sensor and the cell surface. With this technique, accuracy in the positioning of the sensor can be achieved down to 5 μm from the surface of a single HUVEC. The dependence of the current measured upon bradykinin stimulation, indicating the flux of NO released from the

	Biological system	Electrochemical method or device	References
Vascular physiology, tissue growth and angiogenesis	Cultured cells	Nickel tetrasulfonated phthalocyanine/nafion modified carbon fiber microelectrodes	Lantoine et al., 1995; Bedioui et al., 1997
		Scanning electrochemical microscopy	Pailleret et al., 2003; Isik et al., 2004; Isik and Schuhmann, 2006
		Microelectrode arrays	Kamei et al., 2004a; Oni et al., 2004; Isik et al., 2005; Patel et al., 2008a; Trouillon et al., 2010a,b, 2011a,b
		Commercial sensor	Sud et al., 2008
	Excised tissue samples	Poly(tetrafluoroethylene) (PTFE) coated platinized platinu electrodes	Lee et al., 2004
		Modified carbon fiber or platinum/iridium microelectrodes	Villeneuve et al., 1998
		Sol-gel derived sensor	Lee et al., 2012
		Commercial sensor	Teng et al., 2008
	In vivo	Modified platinum/iridium electrodes	Griveau et al., 2007, 2009
	Blood and plasma	PTFE modified platinum electrode	Cha et al., 2005, 2009; Cha and Meyerhoff, 2006; Hwang et al., 2008
		Microfluidic system	Huang et al., 2011
Neuronal mediation	Cultured cells	Microelectrode arrays and multianalyte sensing	Castillo et al., 2005; Wartelle et al., 2005
	Excised tissue samples	p-eugenol/nafion modified carbon fiber microelectrodes	Patel et al., 2006, 2010a
		o-phenylenediamine/nafion modified carbon fiber microelectrodes	Ledo et al., 2005
		Platinized carbon fiber microelectrodes	Amatore et al., 2006a; Rancillac et al., 2006
	Gastrointestinal tissue samples	Synthetic diamond microelectrodes	Patel et al., 2008b, 2010b; MacEachern et al., 2011
	In vivo	Modified carbon fiber microelectrodes	Lourenço et al., 2011; Santos et al., 2011
		Modified platinum microelectrodes	Koh et al., 2008; Lee et al., 2010
		Porous platinum nanoelectrode	Jo et al., 2011a,b
		Commercial sensor	Yang et al., 2008
Immune response and oxidative stress	Single cells	Platinized carbon fiber microelectrodes	Amatore et al., 2000, 2001, 2006b, 2008a,b,c, 2010a,b; Arbault et al., 2004; Verchier et al., 2007; Ferreira et al., 2009; Tapsoba et al., 2009; Filipovíc et al., 2010; Hu et al., 2010; Bernard et al., 2012
		Modified carbon fiber microelectrodes	Porterfield et al., 2001
		Platinized carbon fiber nanoelectrodes	Wang et al., 2012
	Population of cultured cells	Porphyrinic NO sensor	Pekarova et al., 2009
		Microelectrode arrays	Kamei et al., 2004b; Trouillon et al., 2012
		Multianalyte sensing	Chang et al., 2005
		Electrochemistry combined with microscopy	Pereira-Rodrigues et al., 2005
		Microfluidic systems	Amatore et al., 2007; Cha et al., 2010

Table 1 Summary of the electrochemical methods used for NO sensing in different samples.

cell, over the distance to the cell can be characterized (Figure 1A). It was later shown that the use of the shear-force-based constant distance mode of the SECM allowed the topology of the cell to be resolved and enabled the positioning of the sensor at its apex. This method enabled the quantitative detection of the amount of NO released after bradykinin stimulation (Isik and Schuhmann, 2006). The authors report that individual HUVECs release from 1.4 fmol to 10 fmol of NO over 200–300 s after exposure to bradykinin.

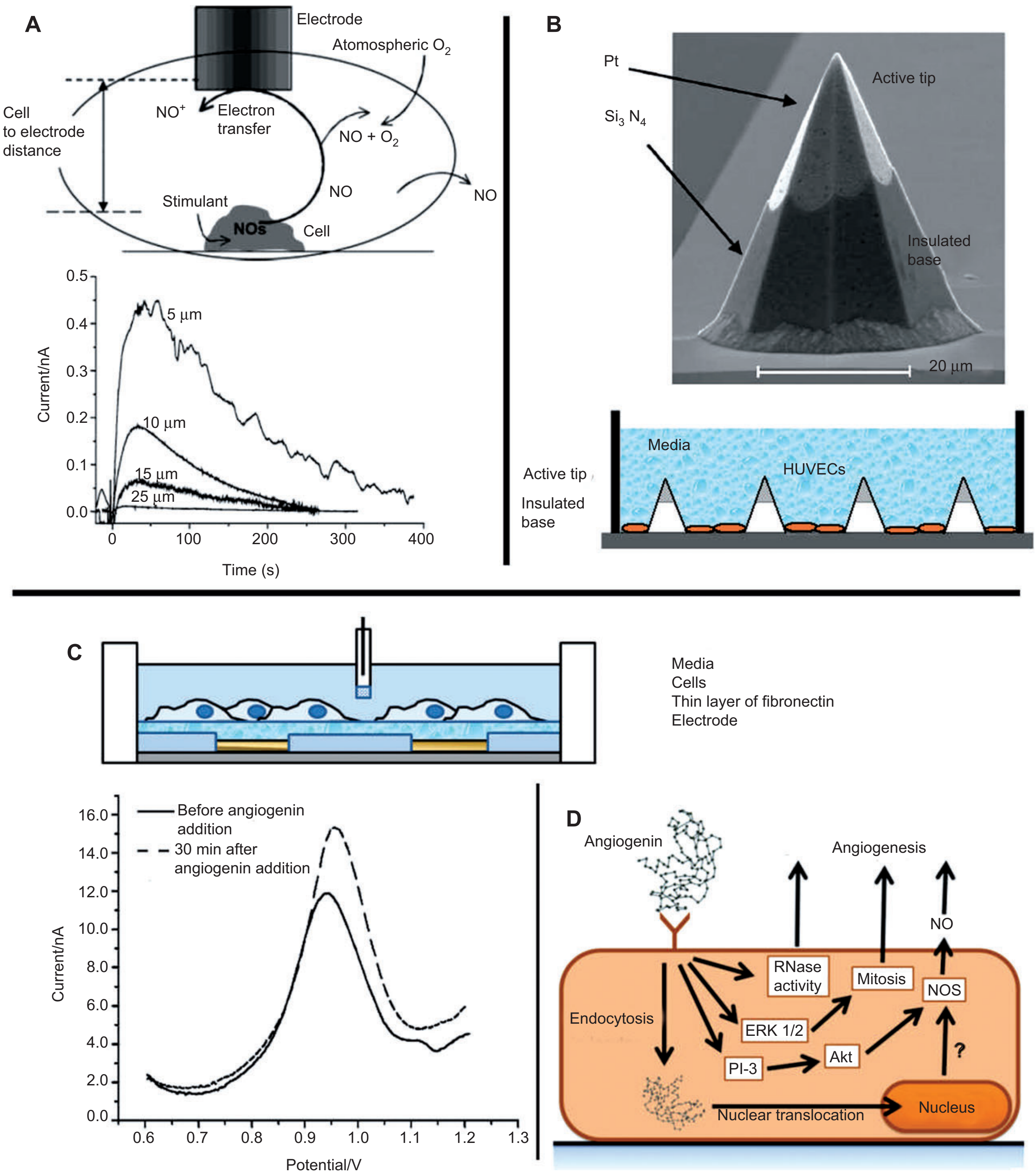

Figure 1 NO sensing for vascular physiology.
(A) SECM for NO detection at a single HUVEC with (top) a scheme summarizing the principle of SECM, showing an electrode positioned above the cell, detecting the released NO and (bottom) typical NO oxidation traces obtained for different cell-to-electrode distances (5 µm, 10 µm, 15 µm and 25 µm) after bradykinin stimulation of HUVECs (adapted with permission from Isik et al. 2004, © 2004 American Chemical Society). (B) Electron micrograph (top) of a three-dimensional electrode array, showing a platinum-coated cone partially insulated in Si_3N_4 and the principle of the measurements (bottom), with cells grown between the cones (adapted with permission from Isik et al. 2005, © 2005 Elsevier). (C) Measurement of angiogenin-induced NO release with (top) a scheme of the setup, showing the cells directly on the fibronectin-coated electrodes and (bottom) a background differential pulse voltammogram performed in the absence of angiogenin (solid line) compared to a second one performed after 30 min of incubation in the presence of angiogenin (dotted lime) showing the increase in oxidative current due to the increase in NO levels (adapted from Trouillon et al. 2010a, © 2010 American Chemical Society). (D) Summary of the angiogenin pathway leading to NO release obtained from microelectrode array experiments (adapted from Trouillon et al. 2011a, © 2011 KSBMB).

Microfabricated devices

Microarrays were also used to detect NO released from cultured cells. The experimental set-up poses a major challenge to the technique: since cells are grown directly on the surface of the device, the electrodes are more susceptible to fouling. In addition, the continuous potential applied during amperometry is not conducive to cell viability. This issue was mitigated by shielding the cell from the electrodes with a layer of biopolymer, such as collagen (Oni et al., 2004). In this article, bradykinin-induced NO release in HUVECs was elicited and used to compare three NO-sensitive electrode coatings. The authors found that electropolymerized films of NiTSPc were more sensitive to NO detection compared to pyrrole-substituted manganese porphyrin or NiTmPyP layers. Patel and coworkers have successfully demonstrated the use of microarrays to simultaneously monitor NO release and oxygen consumption in fibroblast cells grown in collagen matrices and activated by growth factors (Patel et al., 2008a).

Although these gel matrices are beneficial, they can unfortunately be a source of diffusional hindrance. To overcome this issue, Isik and coworkers designed a three-dimensional microelectrode array, shown in Figure 1B (Isik et al., 2005). This array features several cone-shaped processes with functionalized NO-sensing tips. This setup ensures that the cells are not directly adhered to the electrode surface. This platform has been used to successfully detect NO release in bradykinin-stimulated HUVECs. HUVECs can also be grown directly on a thinner of layer poly-l-lysine/poly-styrene-sulfonate. Kamei and coworkers have used this film on gold surfaces to study the effects of various pharmaceutical agents on NO release in HUVECs (Kamei et al., 2004a).

In vivo and *ex vivo* tissue measurements

For tissue-based investigations, NO sensors have been prepared with platinized platinum electrodes housed behind a microporous PTFE gas-permeable membrane. Since NO is deeply involved in kidney physiology, it regulates post-glomerular perfusion and vascular dynamics. Lee and coworkers have used these sensors to detect NO in pig kidney slices (Lee et al., 2004). NO release was evoked by treating the tissue sections with arginine homopolymers. As expected, the amount of NO release correlated with the length of the polymer. This result indicates a fast intracellular translocation and protease cleavage of these polymers into arginine, which enhances NO synthesis. Histamine-induced NO release in perfused pig arteries was used as a model to compare the efficacy of different NO sensors (Villeneuve et al., 1998). The efficacies of a platinum-iridium alloy-coated electrode and a nickel porphyrin/nafion-coated carbon fiber electrode have been investigated in tissue. These modified electrodes are both able to detect NO in tissue, however, the platinum-iridium sensor did not provide sufficient selectivity and was very sensitive to nitrites.

Myocardial ischemia, followed by reperfusion of the tissue, can lead to damages to the tissue. This issue is of particular relevance to cardiac surgery, and hypothermia is usually considered to provide good protection to the myocardium during heart operations. To get a better understanding of the processes leading to this cryoprotection, the levels of NO during ischemia were measured with an integrated sol-gel-derived NO microsensor inserted into perfused hearts from rats (Lee et al., 2012). The effect of hypothermic treatment was investigated. It was found that during the ischemic period, the NO concentration decreases overall, but that the tissues that underwent the hypothermic treatment still release more NO. Promoting NO formation may therefore be a good candidate in the prevention of ischemic injury.

In situ sensing of exogenous NO, injected as authentic NO solutions or produced *in situ* by decomposition of PAPA-NONOate, was performed in tumor-bearing mice (Griveau et al., 2007) with chemically-modified platinum/iridium electrodes. Detection of NO derived from intraperitoneally injected nitroglycerin in the tumor of living mice has also been reported (Griveau et al., 2009). These experiments indicate the possibility of using electrochemical NO sensing for *in vivo* studies of carcinogenesis.

Blood, plasma and blood cells

Several vascular pathologies, such as atherosclerosis, hypertension and diabetes (Hwang et al., 2008) can arise from the impairment of NO synthesis or release in blood, thus motivating the design of analytical methods specific to blood. NO can be stored in the blood as RSNO. Anomalies associated with the conversion of NO to RSNO or *vice versa* can lead to pathologies. The detection of this NO precursor has therefore raised some interest and involves the use of a modified NO sensor (i.e., a PTFE-modified platinum electrode). This device is combined with a NO catalytic generating layer producing NO from RSNO (Cha et al., 2005, 2009; Cha and Meyerhoff, 2006; Hwang et al., 2008). Direct detection of these compounds in plasma or whole blood has been performed as a proof of concept. Recently, this design was improved by combining the technique with microfluidics (Huang et al., 2011). Huang and coworkers have recently designed a flow injection system that mixes the plasma samples and a stimulation solution, containing 3-3′dipropionicdiselenide and glutathione. The production of NO is detected downstream with a NO sensor.

Although collagen does not activate NO production in adherent cells, platelets are sensitive to collagen, a biomarker associated with wounds. From electrochemical measurements of collagen-induced NO release in platelets, Bedioui and coworkers found that NO synthesis is independent of the levels of cytosolic calcium (Lantoine et al., 1995; Bedioui et al., 1997). It is suggested by the authors that this release mediates thrombus and tissue growth, as well as local control of the vascular tone.

Recent developments

This section focuses on more recent breakthroughs, particularly in the past 3 years, which have contributed to improving the electrochemical detection of NO associated with vascular physiology, tissue growth and angiogenesis.

For microarray-based detection devices, the development and use of fibronectin-coated microelectrode arrays instead of traditional collagen coated electrodes has improved NO detection (Figure 1C). Fibronectin was found to efficiently protect the sensor from protein biofouling (Trouillon et al., 2009a,b). In addition, the cell viability issue associated with continuous potential amperometry was overcome by using two series of differential pulse voltammograms and comparing their relative variations (Figure 1C). As a proof of concept, this device was used to investigate the effect of vascular endothelial growth factor on NO release (Trouillon et al., 2010b). The study shows that this growth factor induces NO synthesis via the phosphatidylinositol-3 (PI-3)/Akt kinase pathway. In a similar study, another angiogenic factor, angiogenin, was found to induce NO release via the phosphorylation of the PI-3/Akt kinase pathway (Trouillon et al., 2010a, 2011a). This activity was found to be independent of the RNase activity of angiogenin (Trouillon et al., 2011b), as shown in Figure 1D. Although the role of the nuclear translocation of angiogenin in this phenomenon has not yet been elucidated, these results support the use of simple, microfabricated electrochemical devices for routine biochemical cell-based assays.

Neuronal mediation

NO is synthesized in neurons (Bredt et al., 1990) and is a critical factor in neuronal communication (Garthwaite et al., 1988). Neurotransmitter release is modulated by nitrergic neurons via the synthesis of NO (Prast and Philippu, 2001), thus encouraging the development of specific sensors for the analysis of NO release in the brain or neuronal samples.

In vitro cell measurements

Bradykinin-stimulated C6 glioma cells have been used as a neuronal cell model to study NO release. These experiments have been performed on microelectrode arrays (Figure 2A) coated with a thick layer of biocompatible gels (Castillo et al., 2005; Wartelle et al., 2005). Wartelle and coworkers used a carbon electrode modified by electrodepositing a NiTSPc film to measure NO levels after exposure to bradykinin and after NOS inhibition with L-NAME (Wartelle et al., 2005). This setup was extended by simultaneously measuring NO and glutamate levels after bradykinin stimulation, as shown in Figure 2B. In this experimental setup, NO was sensed with a platinized gold electrode coated with a NiTmPyP layer and glutamate was monitored with a gold electrode modified with a horseradish peroxidase/glutamate oxidase hydrogel (Castillo et al., 2005).

Ex vivo measurements in excised tissues

Microelectrode-based NO detection schemes have also been used to study primary neurons on the single cell level.

The detection of NO release from neurons in the isolated brain of the pond snail, *Lymnaea stagnalis*, has been performed with a poly-eugenol/nafion-modified carbon fiber microelectrode (Patel et al., 2006). In this study, NO release was induced by high levels of K^+ and Ca^{2+} from the cell bodies of two neurons, the B2 buccal motor neuron and the cerebral giant cell, as shown in Figure 2C. NO releases in cerebral giant cell from young and old snails were then compared (Patel et al., 2010a). The time taken for NO release to reach a steady state increased with age. This result possibly indicates a hindrance in NOS activity or scavenging of NO by secondary species during aging.

NO detection has been performed in rat brain slices; after electrically stimulating the white matter (Shibuki, 1990) or by perfusing N-methyl D-aspartate (NMDA) over the samples (Ledo et al., 2005). These results demonstrate the ability to study NO as a mediator in an intact neuronal network.

It has been suggested that NO is released by neurons to regulate local blood flow, especially during times of high

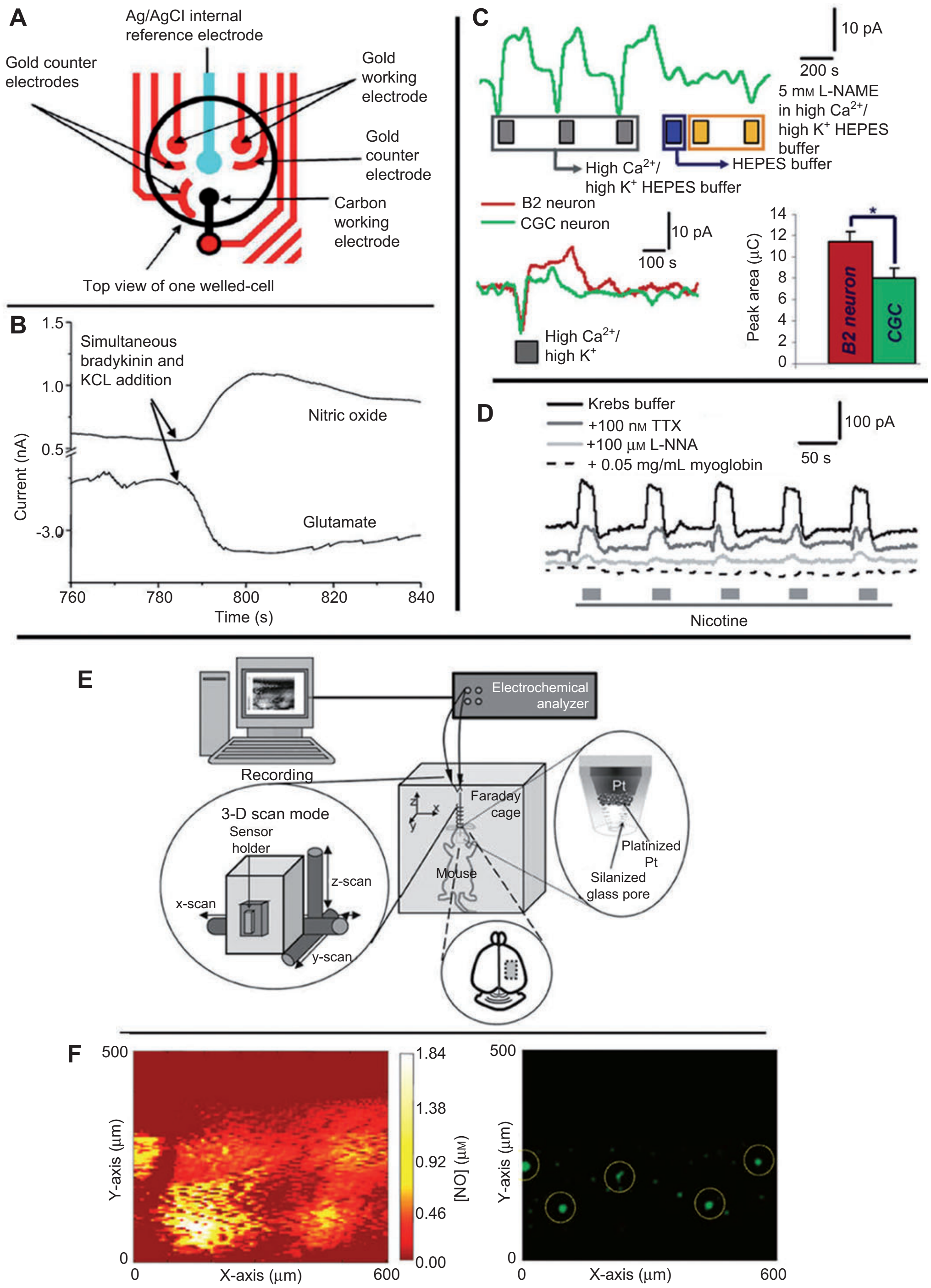

Figure 2 NO sensing for neuronal mediation.
(A) Layout of a microelectrode array showing the carbon working electrodes, and two auxiliary gold electrodes (adapted from Wartelle et al. 2005, © 2005 Elsevier). (B) Simultaneous detection of NO and glutamate release from C6 glioma cells with a modified microelectrode array (adapted from Castillo et al. 2005, © 2005 Elsevier). (C) NO release in the pond snail *Lymnaea stagnalis* in (top) a B2 cell from a young snail, where NO release is evoked by high levels of K^+ and Ca^{2+}, thus leading to an increase in the measured current, which is then abolished by L-NAME and (bottom) in a B2 cell and a cerebral giant cell, showing that the B2 neuron releases more NO than the cerebral giant cell (adapted with permission from Patel et al. 2006, © 2006 American Chemical Society). (D) NO release from the excised guinea pig enteric wall triggered by 20 s perfusions of 1 mM nicotine (indicated by the gray bars) showing the scavenging of NO with myoglobin or inhibition of NO release with tetrodotoxin or N-nitro-L-arginine (adapted from Patel et al. 2008b, © 2008 Blackwell Publishing). (E) SECM setup used for the *in vivo* spatial detection of NO, showing the moving stage and a diagram of the nanopore electrode (adapted from Jo et al. 2011b, © 2011 American Chemical Society). (F) Typical X–Y map of NO release at the surface of a mouse brain obtained with SECM (left) and corresponding confocal nNOS images (right) showing that NO release is correlated with the presence of nNOS containing cells (adapted from Jo et al. 2011b, © 2011 American Chemical Society).

neural activity, which induces high metabolic demands. This phenomenon, known as functional hyperemia, has been observed with platinized carbon fiber microelectrodes in rat cerebellar slices (Amatore et al., 2006a). In this case, NO release was triggered by doping the culture media with NMDA. This method was specifically implemented in order to deconvolute the glutaminergic pathway leading to the neuronal control of microvascular tone (Rancillac et al., 2006). Both glutamate and NMDA induced NO release, as indicated by the increase in oxidative current measured by the sensor. However, glutamate constricted the surrounding vasculature while NMDA dilates this same tissue. These results indicate that cerebellar stellate and Purkinje cells can respectively dilate or constrict microvessels, thus stressing the control of neurons over its neighboring vasculature.

Neuronal mediation in the gastrointestinal tract

Several innovations have recently been reported in neuronal-based investigations dealing with intestinal tissue samples. In the neuronal control of the gut, NO inhibits gastrointestinal motility by relaxing the muscle layers, but its exact role has not been fully elucidated. One of these innovations is the use of BDD electrodes. BDDs have been shown to be effective at detecting NO in freshly excised sections of ileum from guinea pigs and colon from mice. Advantageously, these electrodes have a high resistance to protein and neurotransmitter fouling (Trouillon and O'Hare, 2010; Trouillon et al., 2011c).

The first study using BDD electrodes for the study of NO release from the gastrointestinal tract demonstrated NO detection in longitudinal muscle myenteric plexus and circular muscle strip preparations (Patel et al., 2008b). In this study, NO was determined to be the molecule sensed because the detected signal disappeared when the electrode potential was decreased to 750 mV, a potential well below that required to oxidize NO. Pharmacologically-based treatments (i.e., NOS inhibitor L-NNA and the NO scavenger myoglobin) were tested to confirm that the analyte detected was indeed NO, as shown in Figure 2D. The ion channel antagonist tetrodotoxin blocked all NO release from the ileum. This highlights the role of ion channels and the neuronal control of NO release in the gut. In an age-based study, it was found that the longitudinal muscle myenteric plexus in a neonatal guinea pig released more NO compared to the same sample tissue in the adult guinea pig. The high density of NOS-containing neurons in the young ileum is believed to contribute to this result (Patel et al., 2010b). Recently, it has been suggested that NO release from neurons and enteric glia can be triggered by nicotinic signaling to regulate epithelial ion transport in segments of mouse colon (MacEachern et al., 2011).

Recent developments

In the past 3 years, studies have reported the heterogeneity of NO release and diffusion in the brain. These studies were performed *in vivo* in the rat using an o-phenylenediamine/nafion-modified carbon fiber microelectrode. It appears that the diffusivity of NO is heterogeneous over the volume of the brain. The authors suggest that lipid-rich structures in various regions of the brain can facilitate NO diffusion (Santos et al., 2011).

The same sensor was used to study *in vivo* the dynamics of NO release after glutaminergic neuronal activation in the hippocampus (Lourenço et al., 2011). By using a cytochrome c modified-conducting polymer, Koh and coworkers designed a microelectrode for *in vivo* measurements of NO in the rat brain (Koh et al., 2008). Using this device, the authors reported that repeated injection of cocaine led to higher NO levels in the striatum via a NMDA receptor-evoked Ca^{2+} influx triggered by D1 receptor stimulation (Lee et al., 2010).

Recently, *in vivo* scanning electrochemical detection of NO in the brain has been reported. In this study, a porous platinum nanoelectrode was inserted into the brain of an anesthetized mouse. NO levels were measured as a function of depth, as the electrode was advanced into the brain tissue until it reached the hippocampus (Jo et al., 2011a). The concentration of NO increased as the sensor was pushed deeper into the hippocampus. SECM was also performed with this electrode at the surface of a living mouse cortex, as shown in Figure 2E (Jo et al., 2011b). Immunohistochemical assays for nNOS revealed a correlation between NO release and nNOS-containing cells (Figure 2F). This research demonstrates the method's ability to precisely map NO release *in vivo*, particularly in the brain.

Immune response and oxidative stress

NO is also a critical component of immune response, inflammation and oxidative stress. Due to the radical nature of NO, high levels of NO can be cytotoxic (Kröncke et al., 1997). During immune response, NO is co-released with superoxide O_2^-. The combination of these two species is responsible for forming extremely cytotoxic compounds, such as peroxynitrite. Peroxynitrous acid, the acid conjugate of peroxynitrite, has also been found to be involved in lipid peroxidation (Radi et al., 1991). This phenomenon is known as oxidative stress. Oxidative stress is particularly relevant to electrochemical detection, since most of the reactive nitrogen and oxygen species (RNS and ROS, respectively) involved in the process are electroactive.

In vitro single cell experiments

Oxidative stress has been studied at the single cell level, by forming an artificial synapse between the cell and the electrode (Amatore et al., 2000). In this process, a platinized carbon fiber microelectrode is positioned above the surface of the cell. There are various methods for stimulating oxidative stress. For example, it has been induced in macrophages, fibroblasts or osteosarcoma cells by mechanical stimulation with a glass pipette, as shown in Figure 3A (Amatore et al., 2006b, 2008b; Hu et al., 2010). The pipette punctures the cell membrane, thus depolarizing it and triggering the co-release of NO and O_2^-. These species then react with each other to produce a toxic mixture of ROS and RNS. The oxidative signature of peroxynitrite can be observed with this system (Amatore et al., 2001).

Immunostimulants such as interferon-γ and lipopolysaccharides are also used to induce oxidative stress (Amatore et al., 2008c). Interferon-γ-stimulated macrophages were used as a model for NO release, and the effect of the presence of background species on the diffusion of NO was studied (Porterfield et al., 2001). In complex biological systems there are a number of species that may interfere with the detection of NO. It has been shown that lipids or proteins, like bovine serum albumin, seriously hindered NO diffusion while small molecules, such as glutathione, did not. This report, in agreement with others (Santos et al., 2011), indicates that macromolecules and tissue structure can alter the diffusivity of NO.

The various components of the cytotoxic cocktail released by a cell were detected based on their different oxidation potentials (Figure 3B). It is therefore possible to estimate the chemical composition of the oxidative burst, and then to calculate the relative amounts of NO and O_2^- released by the cell, as shown in Figures 3C and 4.

This method has been used to disentangle the effect of drugs or pathologies on the relative amounts of ROS and RNS released during oxidative stress. It has been shown that osteosarcoma cells release higher levels of NO (Hu et al., 2010), thus leading to the formation of diseased bones. In contrast, cancer-prone xeroderma pigmentosum fibroblasts synthesize less NO compared to the amount of O_2^- released (Arbault et al., 2004). It was also reported that, in PLB-985 cells, NOS can provide a pseudo-NADPH oxidase activity in cells deprived of this enzyme (Verchier et al., 2007). Finally the effect of ascorbic acid on the release of NO was investigated, and the pro-oxidant ability of ascorbic acid in RAW 264.7 macrophages, at low concentrations, was observed (Amatore et al., 2008a).

The spatial distribution of RNS and ROS release sites across the surface of a mechanically-stimulated fibroblast was achieved using electrochemical sensors to triangulate their positions (Amatore et al., 2008b). RNS and ROS release was limited to a 15 μm radius region around the puncture site. These regions experience localized membrane depolarization that induces spatially-limited ionic currents, and therefore RNS and ROS release.

In vitro measurements from a population of cells

Integrated NO sensors have been used to investigate NO release. These sensors offer the advantage of having a complete electrochemical setup in a single device. Using an integrated NO sensor, Pereira-Rodrigues and coworkers measured the release of NO in a population of cultured cells (Pereira-Rodrigues et al., 2006). This particular system has also been used to study NO release after stimulating human U937 promonocytic cells with phorbol myristate acetate. Similarly, a porphyrinic NO sensor was able to measure NO release induced by lipopolysaccharides in RAW 264.7 macrophages in real-time (Pekarova et al., 2009).

Microfabricated systems

Microfabricated devices have also been successfully used. Kamei and coworkers have studied NO release in lipopolysaccharide and interferon-γ-stimulated RAW 264.7 macrophages cultured on a polyion-coated gold microelectrode array (Kamei et al., 2004b).

Simultaneous detection of NO and other electrochemical active species can be achieved with the help of microfabricated devices. Chang and coworkers have demonstrated this by simultaneously detecting NO and O_2^- released from phorbol myristate acetate-stimulated A172 glioblastoma

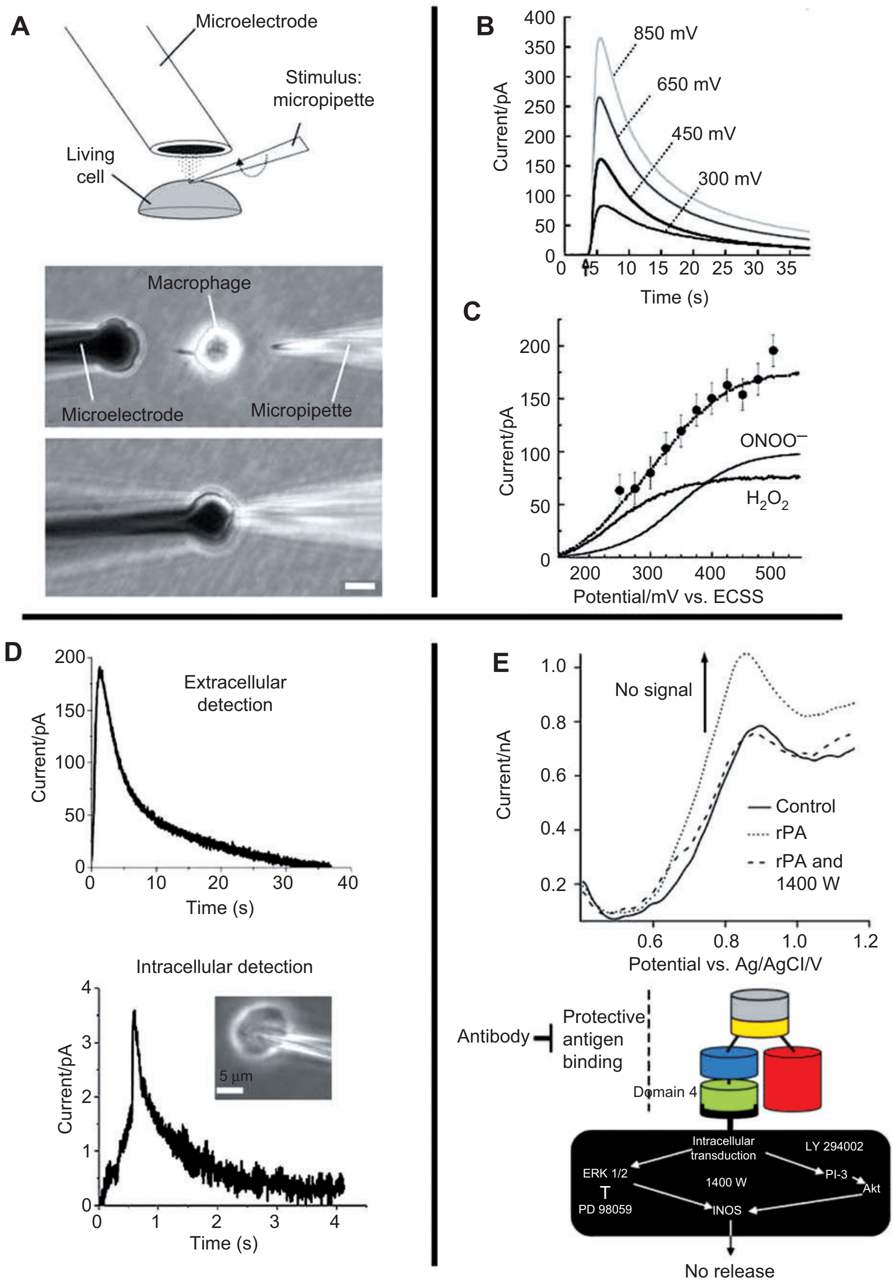

Figure 3 NO sensing for immune response and oxidative stress.
(A) Setup for mechanical stimulation of a single macrophage showing (top) the principle of the experiment where a platinized electrode is held over a single macrophage, and the different components of the system before (middle) and after (bottom) positioning the electrode and the stimulating pipette over the cell (scale bar: 10 µm, adapted with permission from Amatore et al. 2006b, © 2006 Wiley-VCH).
(B) Typical responses from mechanically-stimulated single macrophages for different electrode potentials, thus allowing quantification of the different species released by the cell (adapted with permission from Amatore et al. 2006b, © 2006 Wiley-VCH). (C) Reconstructed steady-state voltammograms from time-current responses obtained from mechanically-stimulated single macrophage for different electrode potentials (each data point corresponds to 30–40 individual responses), compared to the voltammograms obtained *in vitro* with the same microelectrodes for solutions of 1.2 mM H_2O_2 and 2.4 mM peroxynitrite $ONOO^-$ in phosphate-buffered saline (PBS) (adapted with permission from Amatore et al. 2006b, © 2006 Wiley-VCH). (D) Intracellular detection of RNS and ROS was performed inside a single macrophage (scale bar: 5 µm), and the levels of RNS and ROS were simultaneously measured inside and outside the cell (adapted with permission from Wang et al. 2012, © 2012 National Academy of Sciences, USA). (E) Microelectrode array-based detection of NO release in macrophages stimulated by a recombinant protective antigen (rPA) from *Bacillus anthracis* (top) based on successive differential pulse voltammograms, showing that the addition of protective antigen induces a rise in oxidative current, abolished by the inducible NOS inhibitor 1400 W and (bottom) activation pathway leading to the release of NO from a macrophage after binding of the antigen to its membrane receptor (adapted from Trouillon et al. 2012, © 2012 Elsevier).

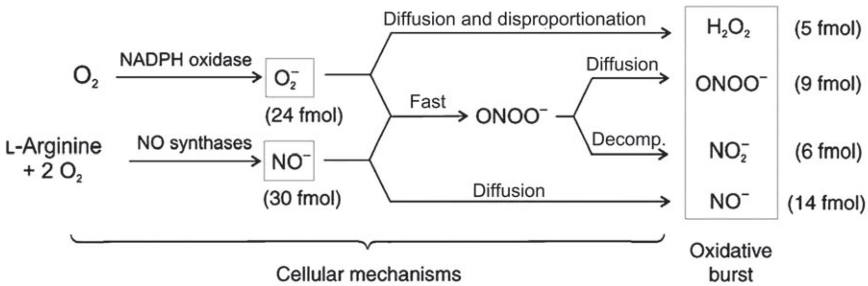

Figure 4 Reaction pathway leading to the oxidative stress release of reactive oxygen and nitrogen species in a mechanically-stimulated macrophage, and quantification of the initial amounts of NO and O_2^- released (adapted with permission from Amatore et al. 2006b, © 2006 Wiley-VCH).

cells (Chang et al., 2005). The release of O_2^- was detected with a cytochrome-c coated-gold electrode and NO detection with a NiTSPc/nafion-coated carbon electrode.

Electrochemistry has also been combined with microscopy. Pereira-Rodrigues and coworkers have designed a 24-well plate with 1 mm NiTSPc/nafion-coated carbon disk electrodes located at the bottom of each well. Oxidative nitrosation was observed with a DAF fluorescent probe. This technique allowed for the simultaneous detection of intracellular and extracellular NO levels (Pereira-Rodrigues et al., 2005).

Amatore and coworkers used a three-electrode setup to detect the release of RNS and ROS from macrophages cultured in a microfluidic chamber. The release of RNS and ROS was triggered by injecting a calcium ionophore into the system and detected amperometrically with a platinized electrode (Amatore et al., 2007). Similarly, another fluidic setup, containing a gold-hexacyanoferrate-coated electrode covered with a Teflon-based gas permeable membrane (Cha et al., 2010) enabled the detection of the release of NO evoked by lipopolysaccharide from a confluent layer of RAW 264.7 macrophages.

Recent developments

In the past three years, new innovations, such as chronoamperometry, and new applications, one of which has helped to explain ancient customs, have expanded the field of NO electrochemical sensing applied to immune systems.

Traditionally, multiple analyte sensing involves the use of multiple electrodes (i.e., at least one electrode for each species detected). Recently, however, Amatore and coworkers have proposed the use of chronoamperometry to rapidly quantify different reactive species released from a single macrophage with a single electrode (Amatore et al., 2010a). In this setup, the electrode oxidation potential is rapidly switched, following a staircase waveform, which allows for the chemical resolution of the RNS and ROS mixture released. Relative temporal variations in the levels these compounds were observed.

The simplicity of amperometric detection with a platinized electrode has led to its use in several pharmaceutical investigations aimed at unraveling oxidative stress. This technique was used to elucidate the ancient Egyptian customs of wearing thick eye makeup. The eye makeup used in ancient Egypt contained non-natural lead chloride, which was found to induce oxidative stress in keratinocytes through the release of Pb^{2+} (Tapsoba et al., 2009). It is believed that the high level of NO induced by this eye makeup helped to prevent infection, bacteria proliferation and illnesses related to the eyes.

Different pro- or antioxidant and azido-based drugs were also tested. A drug used in cancer treatment, β-lapachone, was tested in RAW 264.7 macrophages (Ferreira et al., 2009). Although initially thought to show only pro-oxidant properties, the antioxidant ability of β-lapachone was observed, leading to a decrease in the amount of NO released after short (i.e., less than an hour) incubations. This sequence of antioxidant and pro-oxidant activities was attributed to the initial chelation of calcium followed by a redox cycling mediating electron transfer to dissolved oxygen and forming radical species.

Artificial superoxide dismutases are potential candidates for curing diseases where hyperinflammation occurs because of their ability to convert O_2^- into hydrogen peroxide. These compounds were also found to scavenge peroxynitrite and sometimes NO to nitrite, thus removing these very reactive, toxic species and converting them into less active compounds (Filipovíc et al., 2010; Bernard et al., 2012).

Finally, the pro-oxidant activity of azidothymidine (AZT), a drug used in HIV treatment, was demonstrated in macrophages (Amatore et al., 2010b). This property was found to be closely related to the presence of the free azide terminal group, thus underlining the role of this azido moiety in AZT-induced oxidative stress.

The use of nanoelectrodes for the detection of NO inside a cell (intracellular detection) has recently been reported and is shown in Figure 3D (Wang et al., 2012). In this report, oxidative stress is measured inside and outside the cell. Recessed platinum nanoelectrodes were platinized under atomic force microscopic control. These electrodes were positioned inside a macrophage. The act of puncturing the macrophage triggered the oxidative stress response. The intracellular response was much shorter and less important than the extracellular response. This suggests that when cytotoxic species leak inside the cell, the cell rapidly eliminates them in order to avoid damage.

Recently, microarrays have been used to investigate the effect of a recombinant protective antigen from *Bacillus anthracis* on macrophages (Trouillon et al., 2012). It was found that this antigen induces NO release in a dose-time-dependent manner and that the response is mediated via the extracellular signal-regulated protein kinases 1 and 2 and PI-3/Akt kinase pathways, after binding of domain 4 of the antigen to the cell receptors (Figure 3E). Furthermore, pre-incubation of the cells with AZT enhanced the evoked NO release. With this system, antigen levels were detected down to 10 ng/ml.

Conclusions

The field of electrochemical detection of NO has grown over the past 20 years, and several significant biological discoveries have been made using this technology in several fields, including vascular physiology, tissue growth, immune response, neuronal communication, pharmacology and inflammation. Once the issues associated with *in situ* sensing have been solved (i.e., fouling, selectivity, etc.), the high sensitivity and fast response times offered by these NO sensors are an ideal complement to the more common biochemical tests. In particular, simple, cheap, microfabricated cell-based electrochemical sensors are expected to hugely facilitate parallel and direct measurements of NO synthesis.

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References

Amatore, C., Arbault, S., Bruce, D., de Oliveira, P., Erard, M., and Vuillaume, M. (2000). Analysis of individual biochemical events based on artificial synapses using ultramicroelectrodes: cellular oxidative burst. Faraday Discuss. *116*, 319–333.

Amatore, C., Arbault, S., Bruce, D., de Oliveira, P., Erard, M., and Vuillaume, M. (2001). Characterization of the electrochemical oxidation of peroxynitrite: relevance to oxidative stress bursts measured at the single cell level. Chem. Eur. J. *7*, 4171–4179.

Amatore, C., Arbault, S., Bouret, Y., Cauli, B., Guille, M., Rancillac, A., and Rossier, J. (2006a). Nitric oxide release during evoked neuronal activity in cerebellum slices: detection with platinized carbon-fiber microelectrodes. ChemPhysChem *7*, 181–187.

Amatore, C., Arbault, S., Bouton, C., Coffi, K., Drapier, J.C., Ghandour, H., and Tong, Y. (2006b). Monitoring in real time with a microelectrode the release of reactive oxygen and nitrogen species by a single macrophage stimulated by its membrane mechanical depolarization. ChemBioChem *7*, 653–661.

Amatore, C., Arbault, S., Chen, Y., Crozatier, C., and Tapsoba, I. (2007). Electrochemical detection in a microfluidic device of oxidative stress generated by macrophage cells. Lab Chip *7*, 233–238.

Amatore, C., Arbault, S., Ferreira, D.C., Tapsoba, I., and Verchier, Y. (2008a). Vitamin C stimulates or attenuates reactive oxygen and nitrogen species (ROS, RNS) production depending on cell state: quantitative amperometric measurements of oxidative bursts at PLB-985 and RAW 264.7 cells at the single cell level. J. Electroanal. Chem. *615*, 34–44.

Amatore, C., Arbault, S., and Erard, M. (2008b). Triangulation mapping of oxidative bursts released by single fibroblasts by amperometry at microelectrodes. Anal. Chem. *80*, 9635–9641.

Amatore, C., Arbault, S., Bouton, C., Drapier, J.C., Ghandour, H., and Koh, A.C. (2008c). Real-time amperometric analysis of reactive oxygen and nitrogen species released by single immunostimulated macrophages. ChemBioChem *9*, 1472–1480

Amatore, C., Arbault, S., and Koh, A.C. (2010a). Simultaneous detection of reactive oxygen and nitrogen species released by a single macrophage by triple potential-step chronoamperometry. Anal. Chem. *82*, 1411–1419.

Amatore, C., Arbault, S., Jaouen, G., Koh, A.C., Leong, W.K., Top, S., Valleron, M.A., and Woo, C.H. (2010b). Pro-oxidant properties of AZT and other thymidine analogues in macrophages: implication of the azido moiety in oxidative stress. ChemMedChem *5*, 296–301.

Arbault, S., Sojic, N., Bruce, D., Amatore, C., Sarasin, A., and Vuillaume, M. (2004). Oxidative stress in cancer prone xeroderma pigmentosum fibroblasts. Real-time and single cell monitoring of superoxide and nitric oxide production with microelectrodes. Carcinogenesis *25*, 509–515.

Archer, S. (1993). Measurement of nitric oxide in biological models. FASEB J. *7*, 349–360.

Bard, A.J. and Faulkner, L.R. (1980). Electrochemical Methods: Fundamentals and Applications (New York, USA: Wiley).

Bedioui, F. and Trévin, S. (1998). Practical aspects and methodological approaches to achieve electrochemical detection of submicromolar NO in biological systems. Biosens. Bioelectron. *13*, 227–230.

Bedioui, F. and Villeneuve, N. (2003). Electrochemical nitric oxide sensors for biological samples – principle, selected examples and applications. Electroanal. *15*, 5–18.

Bedioui, F., Trévin, S., and Devynck, J. (1996). Chemically modified microelectrodes designed for the electrochemical determination of nitric oxide in biological systems. Electroanal. *8*, 1085–1091.

Bedioui, F., Trévin, S., Dvynck, J., Lantoine, F., Brunet, A., and Devynck, M.A. (1997). Elaboration and use of nickel planar macrocyclic complex-based sensors for the direct electrochemical measurement of nitric oxide in biological media. Biosens. Bioelectron. *12*, 205–212.

Bedioui, F., Quinton, D., Griveau, S., and Nyokong, T. (2010). Designing molecular materials and strategies for the electrochemical detection of nitric oxide, superoxide and peroxynitrite in biological systems. Phys. Chem. Chem. Phys. *12*, 9976–9988.

Bernard, A.S., Giroud, C., Ching, H.Y., Meunier, A., Ambike, V., Amatore, C., Collignon, M.G., Lemaître, F., and Policar, C. (2012). Evaluation of the anti-oxidant properties of a SOD-mimic Mn-complex in activated macrophages. Dalton Trans. *41*, 6399–6403.

Bredt, D.S., Hwang, P.M., and Snyder, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature *347*, 768–770.

Brown, F.O., Finnerty, N.J., and Lowry, J.P. (2009). Nitric oxide monitoring in brain extracellular fluid: characterisation of Nafion®-modified Pt electrodes *in vitro* and *in vivo*. Analyst *134*, 2012–2020.

Brunet, A., Pailleret, A., Devynck, M., Devynck, J., and Bedioui, F. (2003). Electrochemical sensing of nitric oxide for biological systems: methodological approach and new insights in examining interfering compounds. Talanta *61*, 53–59.

Castillo, J., Isik, S., Blöchl, A., Pereira-Rodrigues, N., Bedioui, F., Csöregi, E., Schuhmann, W., and Oni, J. (2005). Simultaneous detection of the release of glutamate and nitric oxide from adherently growing cells using an array of glutamate and nitric oxide selective electrodes. Biosens. Bioelectron. *20*, 1559–1565.

Cha, W. and Meyerhoff, M.E. (2006). S-Nitrosothiol detection *via* amperometric nitric oxide sensor with surface modified hydrogel layer containing immobilized organoselenium catalyst. Langmuir *22*, 10830–10836.

Cha, W., Lee, Y., Oh, B.K., and Meyerhoff, M.E. (2005). Direct detection of S-nitrosothiols using planar amperometric nitric oxide sensor modified with polymeric films containing catalytic copper species. Anal. Chem. *77*, 3516–3524.

Cha, W., Anderson, M.R., Zhang, F., and Meyerhoff, M.E. (2009). Amperometric S-nitrosothiol sensor with enhanced sensitivity based on organoselenium catalysts. Biosen. Bioelectron. *24*, 2441–2446.

Cha, W., Tung, Y.C., Meyerhoff, M.E., and Takayama, S. (2010). Patterned electrode-based amperometric gas sensor for direct nitric oxide detection within microfluidic devices. Anal. Chem. *82*, 3300–3305.

Chang, S.C., Pereira-Rodrigues, N., Henderson, J.R., Cole, A., Bedioui, F., and McNeil, C.J. (2005). An electrochemical sensor array system for the direct, simultaneous *in vitro* monitoring of nitric oxide and superoxide production by cultured cells. Biosens. Bioelectron. *21*, 917–922.

Chng, E.L. and Pumera, M. (2012). Nanographite impurities in carbon nanotubes: their influence on the oxidation of insulin, nitric oxide, and extracellular thiols. Chem. Eur. J. *18*, 1401–1407.

Ciszewski, A. and Milczarek, G. (2003). Electrochemical detection of nitric oxide using polymer modified electrodes. Talanta *61*, 11–26.

Deng, X., Wang, F., and Chen, Z. (2010). A novel electrochemical sensor based on nano-structured film electrode for monitoring nitric oxide in living tissues. Talanta *82*, 1218–1224.

Ferreira, D., Tapsoba, I., Arbault, S., Bouret, Y., Alexandre Moreira, M.S., Ventura Pinto, A., Goulart, M.O., and Amatore, C. (2009). *Ex vivo* activities of β-lapachone and α-lapachone on macrophages: a quantitative pharmacological analysis based on amperometric monitoring of oxidative bursts by single cells. ChemBioChem *10*, 528–538.

Filipovíc, M.R., Koh, A.C., Arbault, S., Niketíc, V., Debus, A., Schleicher, U., Bogdan, C., Guille, M., Lemaître, F., Amatore, C., et al. (2010). Striking inflammation from both sides: manganese (II) pentaazamacrocyclic SOD mimics act also as nitric oxide dismutases: a single-cell study. Angew. Chem. Int. Edit. *49*, 4228–4232.

Frost, M.C., Reynolds, M.M., and Meyerhoff, M.E. (2005). Polymers incorporating nitric oxide releasing/generating substances for improved biocompatibility of blood-contacting medical devices. Biomaterials *26*, 1685–1693.

Garthwaite, J., Charles, S.L., and Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature *336*, 385–388.

Griveau, S., Dumézy, C., Séguin, J., Chabot, G., Scherman, D., and Bedioui, F. (2007). *In vivo* electrochemical detection of nitric oxide in tumor-bearing mice. Anal. Chem. *79*, 1030–1033.

Griveau, S., Séguin, J., Scherman, D., Chabot, G., and Bedioui, F. (2009). *In vivo* electrochemical detection of nitroglycerin-derived nitric oxide in tumor-bearing mice. Electroanal. *21*, 631–634.

Gunasekara, D.B., Hulvey, M.K., Lunte, S.M., and da Silva, J.A. (2012). Microchip electrophoresis with amperometric detection for the study of the generation of nitric oxide by NONOate salts. Anal. Bioanal. Chem. *403*, 2377–2384.

Hetrick, E.M. and Schoenfisch, M.H. (2009). Analytical chemistry of nitric oxide. Annu. Rev. Anal. Chem. *2*, 409–433.

Hu, R., Guille, M., Arbault, S., Lin, C.J., and Amatore, C. (2010). *In situ* electrochemical monitoring of reactive oxygen and nitrogen species released by single MG63 osteosarcoma cell submitted to a mechanical stress. Phys. Chem. Chem. Phys. *12*, 10048–10054.

Huang, C., Brisbois, E., and Meyerhoff, M.E. (2011). Flow injection measurements of S-nitrosothiols species in biological samples using amperometric nitric oxide sensor and soluble organoselenium catalyst reagent. Anal. Bioanal. Chem. *400*, 1125–1135.

Hwang, S., Cha, W., and Meyerhoff, M.E. (2008). Amperometric nitrosothiol sensor using immobilized organoditelluride species as selective catalytic layer. Electroanal. *20*, 270–279.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., and Chaudhuri, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA *84*, 9265.

Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E., and Byrns, R.E. (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proc. Natl. Acad. Sci. USA *90*, 8103–8107.

Isik, S. and Schuhmann, W. (2006). Detection of nitric oxide release from single cells by using constant-distance-mode scanning electrochemical microscopy. Angew. Chem. Int. Edit. *45*, 7451–7454.

Isik, S., Etienne, M., Oni, J., Blöchl, A., Reiter, S., and Schuhmann, W. (2004). Dual microelectrodes for distance control and detection of nitric oxide from endothelial cells by means of scanning electrochemical microscope. Anal. Chem. *76*, 6389–6394.

Isik, S., Berdondini, L., Oni, J., Blöchl, A., Koudelka-Hep, M., and Schuhmann, W. (2005). Cell-compatible array of three-dimensional tip electrodes for the detection of nitric oxide release. Biosens. Bioelectron. *20*, 1566–1572.

Jo, A., Do, H., Jhon, G.J., Suh, M., and Lee, Y. (2011a). Real-time evaluation of nitric oxide (NO) levels in cortical and hippocampal areas with a nanopore-based electrochemical NO sensor. Neurosci. Lett. *10*, 22–25.

Jo, A., Do, H., Jhon, G.J., Suh, M., and Lee, Y. (2011b). Electrochemical nanosensor for real-time direct imaging of nitric oxide in living brain. Anal. Chem. *83*, 8314–8319.

Kamei, K., Haruyama, T., Mie, M., Yanagida, Y., Aizawa, M., and Kobatake, E. (2004a). The construction of endothelial cellular biosensing system for the control of blood pressure drugs. Biosens. Bioelectron. *19*, 1121–1124.

Kamei, K., Mie, M., Yanagida, Y., Aizawa, M., and Kobatake, E. (2004b). Construction and use of an electrochemical NO sensor in a cell-based assessing system. Sensor. Actuat. B Chem. *99*, 106–112.

Khan, M.T. and Furchgott, R.F. (1987). Additional evidence that endothelium-derived relaxing factor is nitric oxide. In: Pharmacology, M.J. Rand and C. Raper, eds. (Amsterdam: Elsevier), pp. 341–344.

Koh, W.C., Rahman, A.M., Choe, E.S., Lee, D.K., and Shim, Y.B. (2008). A cytochrome c modified-conducting polymer microelectrode for monitoring *in vivo* changes in nitric oxide. Biosens. Bioelectron. *23*, 1374–1381.

Kojima, H., Nakatsubo, N., Kikuchi, K., Kawahara, S., Kirino, Y., Nagoshi, H., Hirata, Y., and Nagano, T. (1998). Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. Anal. Chem. *70*, 2446–2453.

Kröncke, K.D., Fehsel, K., and Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection – how, why, when, and where? Nitric Oxide Biol. Ch. *1*, 107–120.

Lantoine, F., Brunet, A., Bedioui, F., Devynck, J., and Devynck, M.A. (1995). Direct measurement of nitric oxide production in platelets: relationship with cytosolic Ca^{2+} concentration. Biochem. Biophys. Res. Commun. *215*, 842–848.

Ledo, A., Barbosa, R.M., Gerhardt, G.A., Cadenas, E., and Laranjinha, J. (2005). Concentration dynamics of nitric oxide in rat hippocampal subregions evoked by stimulation of the NMDA glutamate receptor. Proc. Natl. Acad. Sci. USA *102*, 17483–17488.

Lee, Y., Yang, J., Rudich, S., Schreiner, R., and Meyerhoff, M. (2004). Improved planar amperometric nitric oxide sensor based on platinized platinum anode. 2: Direct real-time measurement of NO generated from porcine kidney slices in the presence of L-arginine, L-arginine polymers, and protamine. Anal. Chem. *76*, 545–551.

Lee, K.L., Koh, W.C., Shim, Y.B., Shim, I., and Choe, E.S. (2010). Repeated cocaine administration increases nitric oxide efflux in the rat dorsal striatum. Psychopharmacol. *208*, 245–256.

Lee, G.J., Chae, S.J., Kim, S.K., Lee, S., Shin, J.H., Kim, W., and Park, H.K. (2012). Real time dynamics of nitric oxide during cardiac ischemia-reperfusion of the rat. Sensor Actuat. B Chem. *161*, 480–485.

Lourenço, C.F., Santos, R., Barbosa, R.M., Gerhardt, G., Cadenas, E., and Laranjinha, J. (2011). *In vivo* modulation of nitric oxide concentration dynamics upon glutamatergic neuronal activation in the hippocampus. Hippocampus *21*, 622–630.

MacEachern, S.J., Patel, B.A., McKay, D.M., and Sharkey, K.A. (2011), Nitric oxide regulation of colonic epithelial ion transport: a novel role for enteric glia in the myenteric plexus. J. Physiol. *589*, 3333–3348.

Malinski, T. and Taha, Z. (1992). Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor. Nature *358*, 676–678.

Malinski, T., Mesaros, S., and Tomboulian, P. (1996). Nitric oxide measurement using electrochemical methods. Methods Enzymol. *268*, 58–69.

Moncada, S. and Higgs, A. (1993). Mechanisms of disease the L-arginine-nitric oxide pathway. N. Engl. J. Med. *329*, 2002–2012.

Moncada, S., Radomski, M.W., and Palmer, R.M. (1988). Endothelium-derived relaxing factor: identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem. Pharmacol. *37*, 2495–2501.

Moncada, S., Palmer, R.M., and Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. *43*, 109–142.

Mowery, K.A., Schoenfisch, M., Saavedra, J.E., Keefer, L.K., and Meyerhoff, M.E. (2000). Preparation and characterization of hydrophobic polymeric films that are thromboresistant *via* nitric oxide release. Biomaterials *21*, 9–21.

Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Symes, J.F., Fishman, M., et al. (1998). Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J. Clin. Invest. *101*, 2567.

Ng, S.R., Guo, C.X., and Li, C.M. (2011). Highly sensitive nitric oxide sensing using three-dimensional graphene/ionic liquid nanocomposite. Electroanal. *23*, 442–448.

Nims, R.W., Cook, J.C., Krishna, M.C., Christodoulou, D., Poore, C., Miles, A.M., Grisham, M.B., and Wink, D.A. (1996). Colorimetric assays for nitric oxide and nitrogen oxide species formed from nitric oxide stock solutions and donor compounds. Methods Enzymol. *268*, 93–105.

Ohshima, H. and Bartsch, H. (1994). Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat. Res. *305*, 253–264.

Oni, J., Pailleret, A., Isik, S., Diab, N., Radtke, I., Blöchl, A., Jackson, M., Bedioui, F., and Schuhmann, W. (2004). Functionalised electrode array for the detection of nitric oxide released by endothelial cells using different NO-sensing chemistries. Anal. Bioanal. Chem. *378*, 1594–1600.

Pailleret, A., Oni, J., Reiter, S., Isik, S., Etienne, M., Bedioui, F., and Schuhmann, W. (2003). *In situ* formation and scanning electrochemical microscopy assisted positioning of NO-sensors above human umbilical vein endothelial cells for the detection of nitric oxide release. Electrochem. Commun. *5*, 847–852.

Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*, 524–526.

Palmer, R.M., Ashton, D.S., and Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature *333*, 664–666.

Park, S.S., Tatum, C.E., and Lee, Y. (2009). Dual electrochemical microsensor for simultaneous measurements of nitric oxide and oxygen: fabrication and characterization. Electrochem. Commun. *11*, 2040–2043.

Patel, B.A. (2011). Electroanalytical approaches to study signaling mechanisms in the gastrointestinal tract. Neurogastroent. Motil. *23*, 595–605.

Patel, B.A., Arundell, M., Parker, K.H., Yeoman, M.S., and O'Hare, D. (2006). Detection of nitric oxide release from single neurons in the pond snail, *Lymnaea stagnalis*. Anal. Chem. *78*, 7643–7648.

Patel, B., Arundell, M., Quek, R., Harvey, S., Ellis, I., Florence, M., Cass, A., Schor, A., and OHare, D. (2008a). Individually addressable microelectrode array for monitoring oxygen and nitric oxide release. Anal. Bioanal. Chem. *390*, 1379–1387.

Patel, B.A., Galligan, J.J., Swain, G.M., and Bian, X. (2008b). Electrochemical monitoring of nitric oxide released by myenteric neurons of the guinea pig ileum. Neurogastroent. Motil. *20*, 1243–1250.

Patel, B.A., Arundell, M., Parker, K.H., Yeoman, M.S., and O'Hare, D. (2010a). Microelectrode investigation of neuroneal ageing from a single identified neurone. Phys. Chem. Chem. Phys. *12*, 10065–10072.

Patel, B.A., Dai, X., Burda, J.E., Zhao, H., Swain, G.M., Galligan, J.J., and Bian, X. (2010b). Inhibitory neuromuscular transmission to ileal longitudinal muscle predominates in neonatal guinea pigs. Neurogastroent. Motil. *22*, 909–e237.

Pekarova, M., Kralova, J., Kubala, L., Ciz, M., Lojek, A., Gregor, C., and Hrbac, J. (2009). Continuous electrochemical monitoring of nitric oxide production in murine macrophage cell line RAW 264.7. Anal. Bioanal. Chem. *394*, 1497–1504.

Pereira-Rodrigues, N., Zurgil, N., Chang, S.C., Henderson, J.R., Bedioui, F., McNeil, C.J., and Deutsch, M. (2005). Combined system for the simultaneous optical and electrochemical monitoring of intra-and extracellular NO produced by glioblastoma cells. Anal. Chem. *77*, 2733–2738.

Pereira-Rodrigues, N., Bedioui, F., Deutsch, A., Zurgil, N., Afrimzon, E., Shafran, Y., and Deutsch, M. (2006). Construction and use of an integrated electrochemical device for the detection of biologically relevant compounds released from non-adherent cells: application for the electrochemical determination of nitric oxide produced by human U937 cells. Electrochem. Commun. *8*, 341–347.

Pontié, M., Gobin, C., Pauporté, T., Bedioui, F., and Devynck, J. (2000). Electrochemical nitric oxide microsensors: sensitivity and selectivity characterisation. Anal. Chim. Acta *411*, 175–185.

Porras Gutierrez, A., Griveau, S., Richard, C., Pailleret, A., Gutierrez Granados, S., and Bedioui, F. (2009). Hybrid materials from carbon nanotubes, nickel tetrasulfonated phthalocyanine and thin polymer layers for the selective electrochemical activation of nitric oxide in solution. Electroanal. *21*, 2303–2310.

Porterfield, D.M., Laskin, J.D., Jung, S.K., Malchow, R.P., Billack, B., Smith, P.J., and Heck, D.E. (2001). Proteins and lipids define the diffusional field of nitric oxide. Am. J. Physiol. Lung C. *281*, L904–L912.

Prast, H. and Philippu, A. (2001). Nitric oxide as modulator of neuronal function. Prog. Neurobiol. *64*, 51–68.

Privett, B.J., Shin, J., and Schoenfisch, M.H. (2010). Electrochemical nitric oxide sensors for physiological measurements. Chem. Soc. Rev. *39*, 1925–1935.

Quinton, D., Girard, A., Kim, L.T., Raimbault, V., Griscom, L., Razan, F., Griveau, S., and Bedioui, F. (2011). On-chip multi-electrochemical sensor array platform for simultaneous screening of nitric oxide and peroxynitrite. Lab Chip *11*, 1342–1350.

Radi, R., Beckman, J.S., Bush, K.M., and Freeman, B.A. (1991). Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. *288*, 481–487.

Rancillac, A., Rossier, J., Guille, M., Tong, X.K., Geoffroy, H., Amatore, C., Arbault, S., Hamel, E., and Cauli, B. (2006). Glutamatergic control of microvascular tone by distinct GABA neurons in the cerebellum. J. Neurosci. *26*, 6997–7006.

Rees, D.D., Palmer, R.M., Schulz, R., Hodson, H.F., and Moncada, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. Br. J. Pharmacol. *101*, 746–752.

Santos, R.M., Lourenço, C.F., Gerhardt, G.A., Cadenas, E., Laranjinha, J., and Barbosa, R.M. (2011). Evidence for a pathway that facilitates nitric oxide diffusion in the brain. Neurochem. Int. *59*, 90–96.

Shibuki, K. (1990). An electrochemical microprobe for detecting nitric oxide release in brain tissue. Neurosci. Res. *9*, 69–76.

Shin, J.H., Privett, B.J., Kita, J.M., Wightman, R.M., and Schoenfisch, M.H. (2008). Fluorinated xerogel-derived microelectrodes for amperometric nitric oxide sensing. Anal. Chem. *80*, 6850–6859.

Stuehr, D.J. (1999). Mammalian nitric oxide synthases. Biochim. Biophys. Acta Bioenerg. *1411*, 217–230.

Sud, N., Wells, S.M., Sharma, S., Wiseman, D.A., Wilham, J., and Black, S.M. (2008). Asymmetric dimethylarginine inhibits HSP90 activity in pulmonary arterial endothelial cells: role of mitochondrial dysfunction. Am. J. Physiol. Cell. Physiol. *294*, C1407–C1418.

Tapsoba, I., Arbault, S., Walter, P., and Amatore, C. (2009). Finding out Egyptian gods secret using analytical chemistry: biomedical properties of Egyptian black makeup revealed by amperometry at single cells. Anal. Chem. *82*, 457–460.

Teng, J.C., Kay, H., Chen, Q., Adams, J.S., Grilli, C., Guglielmello, G., Zambrano, C., Krass, S., Bell, A., and Young, L.H. (2008). Mechanisms related to the cardioprotective effects of protein kinase C epsilon (PKC ε) peptide activator or inhibitor in rat ischemia/reperfusion injury. Naunyn Schmiedeberg's Arch. Pharmacol. *378*, 1–15.

Trouillon, R. and O'Hare, D. (2010). Comparison of glassy carbon and boron doped diamond electrodes: resistance to biofouling. Electrochim. Acta *55*, 6586–6595.

Trouillon, R., Cheung, C., Patel, B.A., and O'Hare, D. (2009a). Comparative study of poly (styrene-sulfonate)/poly (L-lysine) and fibronectin as biofouling preventing layers in dissolved oxygen electrochemical measurements. Analyst *134*, 784–793.

Trouillon, R., Combs, Z., Patel, B.A., and O'Hare, D. (2009b). Comparative study of the effect of various electrode membranes on biofouling and electrochemical measurements. Electrochem. Commun. *11*, 1409–1413.

Trouillon, R., Kang, D.K., Park, H., Chang, S.I., and O'Hare, D. (2010a). Angiogenin induces nitric oxide synthesis in endothelial cells through PI-3 and Akt kinases. Biochemistry *49*, 3282–3288.

Trouillon, R., Cheung, C., Patel, B.A., and O'Hare, D. (2010b). Electrochemical study of the intracellular transduction of vascular endothelial growth factor induced nitric oxide synthase activity using a multi-channel biocompatible microelectrode array. Biochim. Biophys. Acta *1800*, 929–936.

Trouillon, R., O'Hare, D., and Chang, S.I. (2011a). An electrochemical functional assay for the sensing of nitric oxide release induced by angiogenic factors. BMB Rep. *44*, 699–704.

Trouillon, R., Kang, D.K., Chang, S.I., and O'Hare, D. (2011b). Angiogenin induces nitric oxide release independently from its RNase activity. Chem. Commun. *47*, 3421–3423.

Trouillon, R., O'Hare, D., and Einaga, Y. (2011c). Effect of the doping level on the biological stability of hydrogenated boron doped diamond electrodes. Phys. Chem. Chem. Phys. *13*, 5422–5429.

Trouillon, R., Williamson, E.D., Saint, R.J., and O'Hare, D. (2012). Electrochemical detection of the binding of Bacillus Anthracis protective antigen (PA) to the membrane receptor on macrophages through release of nitric oxide. Biosens. Bioelectron. *38*, 138–144.

Verchier, Y., Lardy, B., Nguyen, M.V., Morel, F., Arbault, S., and Amatore, C. (2007). Concerted activities of nitric oxide synthases and NADPH oxidases in PLB-985 cells. Biochem. Biophys. Res. Commun. *361*, 493–498.

Villeneuve, N., Bedioui, F., Voituriez, K., Avaro, S., and Vilaine, J. (1998). Electrochemical detection of nitric oxide production in perfused pig coronary artery: comparison of the performances of two electrochemical sensors. J. Pharmacol. Toxicol. *40*, 95–100.

Wang, Y., Noël, J.M., Velmurugan, J., Nogala, W., Mirkin, M.V., Lu, C., Guille Collignon, M., Lemaître, F., and Amatore, C. (2012). Nanoelectrodes for determination of reactive oxygen and nitrogen species inside murine macrophages. Proc. Natl. Acad. Sci. USA *109*, 11534–11539.

Wartelle, C., Schuhmann, W., Blöchl, A., and Bedioui, F. (2005). Integrated compact biocompatible hydrogel-based amperometric sensing device for easy screening of drugs involved in nitric oxide production by adherent cultured cells. Electrochim. Acta *50*, 4988–4994.

Wisniewski, N. and Reichert, M. (2000). Methods for reducing biosensor membrane biofouling. Colloid. Surface B *18*, 197–219.

Wisniewski, N., Moussy, F., and Reichert, W.M. (2000). Characterization of implantable biosensor membrane biofouling. Fresenius J. Anal. Chem. *366*, 611–621.

Wu, J.F., Xu, M.Q., and Zhao, G.C. (2010). Graphene-based modified electrode for the direct electron transfer of Cytochrome c and biosensing. Electrochem. Commun. *12*, 175–177.

Xu, X., Yang, L., Jiang, S., Hu, Z., and Liu, S. (2011). High reaction activity of nitrogen-doped carbon nanotubes toward the electrooxidation of nitric oxide. Chem. Commun. *47*, 7137–7139.

Yang, Y., Ke-Zhou, L., Ning, G.M., Wang, M.L., and Zheng, X.X. (2008). Dynamics of nitric oxide and peroxynitrite during global brain ischemia/reperfusion in rat hippocampus: NO-sensor measurement and modeling study. Neurochem. Res. *33*, 73–80.

Ye, X., Rubakhin, S.S., and Sweedler, J.V. (2008). Detection of nitric oxide in single cells. Analyst *133*, 423–433.

Ye, X., Xie, F., Romanova, E.V., Rubakhin, S.S., and Sweedler, J.V. (2009). Production of nitric oxide within the *Aplysia californica* nervous system. ACS Chem. Neurosci. *1*, 182–193.

Yoshimura, T., Yokoyama, H., Fujii, S., Takayama, F., Oikawa, K., and Kamada, H. (1996). *In vivo* EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice. Nat. Biotechnol. *14*, 992–994.

Zheng, D., Hu, C., Peng, Y., Yue, W., and Hu, S. (2008). Noncovalently functionalized water-soluble multiwall-nanotubes through azocarmine B and their application in nitric oxide sensor. Electrochem. Commun. *10*, 90–94.

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